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Comprehensive Assignment of Mass Spectral Signatures from Individual *Bacillus atrophaeus* Spores in Matrix-Free Bioaerosol Mass Spectrometry

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Abstract

We have conducted studies to fully characterize the mass spectral signature of individual *Bacillus atrophaeus*, previously known as *Bacillus subtilis var niger* or *Bacillus globigii*, spores obtained in matrix-free bioaerosol mass spectrometry (BAMS). Mass spectra of spores grown in unlabeled, ^{13}C-labeled and ^{15}N-labeled growth media are used to determine the number of carbon and nitrogen atoms associated with each mass peak. To determine the parent ion structure associated with fragment ions present in the spore spectra, the mass-to-charge (m/z) fragmentation pattern of several chemical standards was obtained.

Our results agree with prior assignments of dipicolinic acid, amino acids and calcium complex ions made in the spore mass spectra. Identity of several previously unidentified mass peaks, key to recognition of *Bacillus* spore by matrix-free BAMS, is revealed. Specifically, a set of fragment peaks in the negative polarity is shown to be consistent with the fragmentation pattern of purine nucleobase containing compounds. The identity of m/z=+74, a marker peak that helps discriminate *Bacillus atrophaeus* from *Bacillus thuringiensis* spores grown in rich medium, is surprisingly a non-descript ion, viz. $[N_1C_4H_{12}]^+$. A probable precursor molecule for the $[N_1C_4H_{12}]^+$ ion observed in spore spectra is trimethyl glycine ($^+N(CH_3)_3CH_2COOH$) that produces a m/z=74 peak in presence of dipicolinic acid.

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INTRODUCTION

Mass spectrometry provides a rapid detection method for characterization of intact microorganisms [1, 2]. By integrating particle based analytical methods and mass spectrometry, a number of single-particle mass spectrometry techniques, such as laser based RSMS[3], PALMS[4], RTAMS[5], ATOFMS[6], LAMPAS[7, 8] and pyrolysis based Py-MS[9], Py–GC–IMS[10], have emerged for detection of individual atmospheric particles in real time. Single micron-sized particle detection of microorganisms is particularly relevant in the detection of pathogenic air-borne cells, for example intentional release of harmful bioaerosols like *Bacillus anthracis*. Therefore, it is not surprising that research efforts to develop single particle mass spectrometry (MS) for bioaerosol detection and identification are being actively pursued by several groups[11-16]. Among the laser based desorption/ionization methods two approaches exist. One utilizes the inherent advantages of matrix assisted laser desorption ionization- mass spectrometry (MALDI-MS) to provide soft ionization method for detection of large biomolecules with least amount of fragmentation for detection of high mass range biomarkers [1, 2, 15, 17-19]. In this method, a matrix molecule is added online to coat the bioaerosol of interest. In demonstration of online matrix addition to bioaerosol detection, Stowers et al. [15] have used 308nm laser on *Bacillus atrophaeus* spore coated with both picolinic acid and sinapinic acid as matrix to produce m/z=1224 Da. This has been attributed to cortex peptidoglycan.

In the second approach[16] a matrix-free laser desorption/ionization method is utilized to eliminate sample preparation, which can impede with in situ real-time particle analysis. Matrix addition can also result in a chemical interaction with the analyte that could substantially alter the nascent analyte mass signature. This might not be desirable in applications that require accurate classification of biological risk agents. Matrix-free laser desorption/ionization has been demonstrated[20] to generate molecular ions with mass-to-charge ranging from a few hundred to 19050 Da from whole bacterial spores (~ 2 x 10^4). Efforts to

provide single cell level sensitivity and detection specificity have resulted in the development of bioaerosol mass spectrometry (BAMS).

BAMS has been demonstrated[16] to provide real-time and reagentless characterization of individual *Bacillus* spores without any sample preparation. The observed mass peaks are mainly at low mass ± 200 Da, presumably due to limitations in the ionization process and mass spectrometer. Mass spectral pattern consists of peaks at characteristic m/z, but with variations in peak intensities. Such variations in spectra are in part due to inhomogenities in laser profile as demonstrated by Steele et al[21]. However, an overall assessment of the average spore spectra allows clear identification of prominent peaks, some of which have been tentatively assigned based on isotope labeling studies[22]. In this study we provide a complete identification of the spore spectra that includes previously unidentified peaks. Our current studies provide a comprehensive comparison of mass spectral signatures for spores grown in ^{13}C and ^{15}N isotopically enriched medium with that of unlabeled medium. In the event of fragment ions involving loss of more than one functional group (e.g. HCN and NH_3) we have run standards containing the fragment (observed ion peak) and the lost functional group (HCN, NH_3) to make an assignment of the sub-structure responsible for the fragment ion. In addition standards were run in presence of dipicolinic acid, found to be present in the spore, for assignment of other fragment ions.

A clear assignment of previously unidentified mass peaks, as presented in this work, establishes their relationship to the spore chemical composition. Furthermore, an evaluation of the robustness of "marker" peaks can only be made once chemically identified. This is especially relevant for peaks that have been used to discriminate *Bacillus* spore species, *B. thuringiensis* and *B. atrophaeus* in our previous studies[16].

EXPERIMENTAL SECTION

Spore Preparation. *B. subtilis var. niger* (ATCC 9372, Dugway Proving Ground, Dugway, UT) cells were grown to mid-log phase in BioExpress cell growth media (Cambridge Isotopes) and then aliquoted in a 1:25 dilution into 75 mL of BioExpress media. The *Bacillus* sporulated in BioExpress sporulation media in a shaker incubator at 32 °C until ~90% of all the cells were refractile (3-4 days). Three such sample sets were made in BioExpress unlabeled, ^{13}C-labeled (98%) and ^{15}N-labeled (98%) cell growth media, respectively. Phase contrast microscopy (Zeiss phase contrast microscope) and spore staining were used to confirm the presence of bacterial spores. Spores were harvested by centrifugation at 8000*g* for 10 min and washed in sterilized double-distilled water. After being washed two times, the spores were reconstituted in double distilled water at concentrations of ~10^8 spores/ml as determined using a Petroff-Hauser counting chamber.

Aerosol Generation. The spore solution was aerosolized at a concentration of ~ 10^6 per ml using a Collison nebulizer with a 1-1.5 L/min flow of clean, dry air. The wet bioaerosol was dried with a diffusion drier containing activated silica gel desiccant and was then piped through copper tubing to the aerosol mass spectrometer inlet. The mean aerodynamic diameter of the aerosol particles at the inlet was determined by aerodynamic sizing (see below) to be 0.96± 0.09 μm, 0.99± 0.09 μm, 0.91± 0.11 μm for spores grown in unlabeled, ^{13}C and ^{15}N labeled growth media. This value is consistent with the value expected for single spores. (The aerodynamic diameter d_a is defined as the diameter of the unit density, ρ_p =1 g/cm^3, sphere that has the same settling velocity as the particle [23]).

Aerosol Time of Flight Spectrometer. The BAMS system is based on a modified TSI ATOFMS (Model 3800) that is being developed[16] for bioaerosol laser desorption/ionization mass spectrometry in our group. An illustration can be found in Gard et al. [6]. Briefly, dried aerosols are sampled at atmospheric background pressure via a 340 μm diameter nozzle into the mass spectrometer to ~ 2.4 Torr pressure. Two skimmers downstream collimate the particle beam and help reduce the pressure to ~10^{-4} Torr in the particle sizing region.

The velocity of the entrained aerosol is determined by measuring its transit time between two orthogonally arranged "sizing" laser (continous-wave 532nm) beams. An orthogonal arrangement of the lasers ensures that only particles that travel straight down the center of the instrument are sized and counted. As particles cross the sizing laser beams two scattered signals separated by particle transit time are produced and detected using photomultiplier tubes.

A conversion to aerodynamic diameter from the measured velocity is used to obtain the size histogram of the aerosol particles. Size calibration is done using monodisperse polystyrene latex beads of known sizes. A trigger pulse based on aerosol velocity is then used to trigger the desorption/ionization (D/I) laser arrival time in the ion source region of the mass spectrometer. The base pressure in the source region is ~ 10^{-7} Torr during data collection. The D/I laser is the fourth harmonic of a Q-switched Nd:YAG (Ultra CFR, Big Sky Laser Technologies, Inc.) laser emitting 266nm radiation with a near Gaussian profile and ~ 5ns pulse width. The laser pulse energy is attenuated using a half-wave plate and polarizer and focused into the ion source using 10cm plano-convex lens. Beam profile measurements (using Spiricon Pyrocam III camera) give a laser beam size of ~ 400 μm (FWHM) in the center plane of the ion source. The average laser pulse energy is 0.22mJ. The laser pulse energy is chosen to be consistent with prior results [16, 21], determined to be the optimum pulse energy for a ~400 μm (FWHM) beam size for *Bacillus atrophaeus* spore. Optimum pulse energy is based on minimal fragmentation and reasonable hit rate (~15%), where hit rate is the number of tracked particles that produce ions upon laser interaction in the ion source and produce a measurable mass spectrum. A full description of the laser fluence dependence of *Bacillus* spores can be found in Steele et al. [21]. Positive and negative ions that are generated by laser-aerosol interaction are extracted in opposite directions using a dual-polarity reflectron time of flight tube to two separate microchannel plate (MCP) detectors for positive and negative ion detection. The detector ion signals are sampled at 500 MHz by a four-channel Cougar digitizer running in extended dynamic range mode (two channels per polarity) for the positive and negative ions. The raw data is analyzed using data import and analysis software developed in our group. An autocalibration

method is used to correct for shot to shot jitter in the time-of-flight spectra. Typically 100-500 spectra are collected. In addition to *Bacillus* spores mass fragmentation pattern of a number of standards were also studied. All chemicals used were obtained from Sigma at the highest available purity. Nucleobase containing compounds studied were adenine, adenosine, adenosine 5'-monophosphate sodium salt, adenosine 5'-diphosphate sodium salt, adenosine 5'-triphosphate sodium salt, cytidine, cytidine monophosphate, cytidine diphosphate, guanosine, guanosine 5'-diphosphate sodium salt, and guanosine 5'-triphosphate sodium salt, β-Nicotinamide adenine dinucleotide reduced dipotassium salt and DNA(source?). In addition trimethyl glycine (betaine) and dipicolinic acid standards and mixture were also studied.

RESULTS & DISCUSSION

Average mass spectra obtained from the mass spectra of individual *Bacillus atrophaeus* spore particles, are shown in Fig. 1 as A, B and C for spores grown in unlabeled, ^{13}C-labeled and ^{15}N-labeled Bioexpress cell growth media, respectively. Although we collect spectra from individual spore particles average spectra is used to characterize the spore mass signature. This reduces the variations[21] observed at the single spectra level and is more representative of the spore mass signature. The overall spore mass signature is similar to that obtained from spores grown in ¼ TY and G medium, reported in a recent publication [16]. The masses observed are in the ~ 200Da range for both the positive and negative ions and classified below under separate chemical groups.

Metal Ions

Metal ions like Na^+ and K^+ are easily identifiable in the spore spectra at m/z= +23 and +39, +41 for labeled and unlabeled growth media and account for the largest ion peaks in the positive spectrum. A set of ion peaks observed at m/z= +57, +66 and +82, based on their number of carbon and nitrogen shifts in the spore mass spectrum, are found to be consistent with calcium ion complexes $[CaOH]^+$, $[CaCN]^+$ and $[CaCNO]^+$. Assignment of these ions as calcium adducts is deduced from high calcium component known to constitute the mineral content of *Bacillus subtilis* spore. We do not see any peak at m/z=20

that would correspond to free Ca^{2+} ion and only observe peaks that correspond to bound Ca^{2+} as in $[CaOH]^+$, $[CaCN]^+$ and $[CaCNO]^+$. In addition to calcium as the predominant divalent ion found in spores [24] magnesium and manganese are also present at elevated concentrations inside a spore[25] and together with calcium can comprise up to 3% of the dry weight of the spore[26]. However we did not detect any peaks for magnesium or manganese either as a free or bound divalent ion in BAMS spore spectra.

Dipicolinic Acid

Peaks observed at m/z=-166,-167 are assigned to DPA ($C_7H_5NO_4$). This assignment is based on observed shifts of 7 mass units in the ^{13}C-labeled and 1 mass unit in ^{15}N-labeled media as shown in Fig. 2 A, B and C. Electron capture negative ion mass spectrometry (ECNIMS) data on dipicolinic acid have reported [27] a strong $M^{\cdot -}$ peak at -167 at relatively low energy, 0.3 eV. Ion molecule reactions between low energy electrons, presumably present in the plume created from laser-spore interaction, and neutral DPA molecule would account for DPA related m/z=-167 ion in the spore mass spectra. The presence of m/z=-166 in the spore mass spectra is indicative of loss of a proton from one of the two carboxylic acid groups (COOH) present in DPA. Further evidence of the presence of DPA in the spore mass spectra is seen from the assignment of the m/z=-122 peak. Observed mass shifts of six units in the ^{13}C-labeled spectra and 1 unit in ^{15}N-labeled spectra, as shown in Fig. 2, match the number of carbon and nitrogen atoms present in $C_6H_4NO_3^-$(m/z=-122) that would result from the loss of a CO_2 group from the deprotonated DPA ion. Both m/z=-166 and -122 peaks are also observed in the mass spectra of pure DPA standard and further support our final assignments that require an assumption of the number of oxygen atoms present in DPA($C_7H_4NO_4^-$) and its fragment ($C_6H_4NO_3^-$) ion.

Amino Acids

Two prominent peaks observed in the spore negative spectra are found at m/z=-173 and -146, respectively. As can be seen from Figs 1 and 2 these peaks show a clear shift to –179,-151 in the ^{13}C-

labeled media and to –177,-147 in the ^{15}N-labeled media, respectively. The deduced assignments inferred from these shifts are consistent with deprotonated arginine ($C_6H_{13}N_4O_2^-$) and glutamic acid ($C_5H_8NO_4^-$), respectively. These assignments also agree with assignments made to BAMS spectra for spores grown in ¼ TY media[16], G media[16] and LB media[22].

Further assignments to the spore spectra in the positive polarity give a peak at m/z=175 that shifts to m/z= 181 and 179 in ^{13}C-labeled and ^{15}N-labeled media. This suggests the m/z=175 to arise from protonated arginine, $C_6H_{14}N_4O_2+H^+$.

The presence of glutamic acid and arginine in the spore spectra is consistent with the reported composition of spore amino acid pools. The very high levels of L-glutamate and L-arginine in *Bacillus subtilis* represent intracellular concentrations of the order of 0.1 M [28]. This is equivalent to ~ 10^7 molecules per spore. In contrast the concentrations of other free amino acids are only 5% of the total pool reported for *Bacillus subtilis* grown in supplemented nutrient broth. Both arginine and glutamic acid when run as pure aerosols standards give a deprotonated and protonated peak [22, 29]. However, no evidence of a protonated glutamate peak is seen in the spore spectrum. In the case of arginine a small contribution from protonated parent ion is observed (see Fig. 1). This result is possibly linked to differences in gas-phase basicities (Arginine= 236 kcal/mol [30] and Glutamic acid = 209 kcal/mol [30]) of the two amino acids favoring a proton transfer from the glutamic acid to arginine in the plume. Standard 1:1 mixtures of arginine: glutamic on aerosolization and ionization have been shown to favor protonation of arginine over glutamic acid [29]. Additionally in the presence of dipicolinic acid the effective ionization probability of arginine and glutamic acid has been shown to increase by as much as an order of magnitude [29]. These results suggest that the two amino acids are in close proximity in the spore, probably in the core along with dipicolinic acid, favoring ion formation. Strong ternary complexes of DPA, Ca^{2+}, and amino acids do exist [31, 32] and a similar network might exist in the spore core. Such a close arrangement would favor effective ionization of the amino acids and allow for proton transfer between glutamate and arginine.

A set of low ion signal peaks (see Figs. 1 & 2) were also observed in the positive average spectrum These have mass to charge values of 30, 70, 72, 86, 88 and are seen to shift by one mass unit to m/z=31, 71,73,87 and 89 in the ^{15}N media and by 1,4,4,5,3 units to 31,74,76,91,91. This result is consistent with a set of amino acids that have lost the carboxyl group from the deprotonated parent ion (a loss of 45 mass units). The assignments obtained for m/z=30, 70, 72, 86, 88 is shown in Fig.1 and consistent with loss of COOH group from glycine, proline, valine, leucine/isoleucine, aspartic acid.

In the positive polarity additional peaks at m/z= 81 and 84 are observed close to the fragmented amino acid peaks. These are found to shift to m/z= 81, 86 and 84, 89 in ^{15}N and ^{13}C growth medium as shown in Fig. 2. Tentative assignments of $C_5OH_5^+$ and $C_5NH_{10}^+$ are found consistent with m/z=81 and 84. The presence of fragmented amino acid peaks in the positive spectrum suggests presence of glycine, proline, valine, leucine/isoleuine and aspartic acid in small amounts in spore. In contrast larger contributions have been observed from decarboxylated amino acids in the vegetative cell spectrum [22].

Phosphates

Peaks observed at m/z=-79, -97 in the spore spectra do not show an isotopic shift in ^{13}C-labeled, ^{15}N-labeled media, as would be expected for PO_3^- and $H_2PO_4^-$ ions. Mass peaks at –79 and –97 are fragment ions that are commonly found in mass spectra of phosphate related compounds. About 45% of the total phosphorous content in *B. subtilis* spores is in nucleic acids. Our results with DNA and free nucleotide standards confirm the expected presence of phosphate fragments at -79 and –97 and support their assignments as PO_3^- and $H_2PO_4^-$ ions in the spore spectra.

Purine nucleobases

The origin of m/z=-134 in the spore spectra obtained using BAMS has been unclear. It was tentatively assigned to aspartic acid [16] as well as potentially linked to DPA complex with Ca^{2+} ion based on desorption-ionization studies with DPA:$Ca(OH)_2$ aerosol mixture [22]. Our current studies provide a more direct assignment on the basis of observed isotopic shifts. We have unambiguously identified the

molecular formula for m/z = -134 to be $C_5N_5H_4^-$. The assignment is depicted in Fig. 2 as m/z=-134, -139, -139 in unlabeled and ^{13}C-labeled, ^{15}N-labeled media, respectively. Furthermore, m/z=-117, -107, -90 have been assigned as $C_5N_4H_1^-$, $C_4N_4H_3^-$, $C_4N_3^-$ based on isotopic shifts to m/z=-122, -111, -94 and m/z=-121, -111, -93 in ^{13}C-labeled and ^{15}-labeled media, and is shown in Fig 2. These assignments indicate a nitrogen rich moiety. Consequently, this assignment rules out aspartic acid, $C_4H_7NO_4$, which has only one nitrogen atom as well as any kind of fragment from a DPA complex with calcium hydroxide, $(C_7H_5NO_4)_2Ca(OH)_2$ which would also have one nitrogen atom. Based on the high number of nitrogen atoms it is likely that m/z=-134, -117,-107,-90 are related to a common parent molecular structure. The assignment of m/z=-134 as $C_5N_4H_1^-$ is found to be consistent with deprotonated adenine, found in free nucleotides, nucleic acids and several co-enzymes. In order to probe the relationship between m/z=-134, -117, -107, -90 and adenine we examined the mass fragmentation pattern of pure adenine standard under our aerosolization and laser desorption-ionization conditions. The spectrum for adenine is shown in Fig. 3. In the negative polarity, m/z=-134, -117, -107, -90 and -65 are clearly observed. This set of peaks is also observed in the *Bacillus* spore spectrum. In addition to adenine we have also examined the mass fragmentation pattern of several adenine (A), guanine (G) and cytosine(C) containing nucleosides, mono, di, tri-phosphate nucleotides, coenzyme and DNA. Representative spectra for guanosine 5'-diphosphate sodium salt and cytidine are shown along with adenine in Fig. 3. Our studies with seven adenine containing molecules that are listed in the experimental section produced a m/z=-134, -117, -107, -90 and -65 spectral pattern. Guanine containing molecules were found to give rise to m/z=-150, -133, -106, -90. In contrast, cytidine exhibits peaks at m/z=-110, -95, -84 and -67 that do not overlap with m/z=-134, -117, -107 and -90. This result indicates that m/z=-134, -117, -107, -90 in the spore spectra largely arise from adenine containing molecules while guanine and its derivatives can also contribute to m/z=-90. We also see evidence of the deprotonated guanine ion in the spore spectrum at m/z =-150. This is depicted in Fig. 2 as $C_5N_5OH_4^-$ ion at m/z= -150, -155, -155 in unlabeled, ^{13}C-labeled and ^{15}N-labeled media, respectively.

The isotopic assignment for the observed fragmentation pattern of m/z=-134, -117, -107, -90, as $C_5N_5H_4^-$, $C_5N_4H_1^-$, $C_4N_4H_3^-$, $C_4N_3^-$ is consistent with adenine-H, adenine-H-NH_3, adenine-H-HCN, and adenine-H-HCN-NH_3 anions. The deprotonated ion, m/z=-134, probably involves loss of the pyrrole hydrogen based on calculated gas-phase acidities for the most acidic hydrogen site [33]. The dissociation pathway for the deprotonated adenine appears to involve loss of NH_3, loss of HCN and loss of NH_3 and HCN. The possible reaction mechanism likely involves pyrimidine ring opening.

The relative contribution of different adenine sources within a spore to its observed mass spectrum cannot be quantified in this work. However, a comparison of typical concentrations of a few key sources such as free adenine nucleotides, i.e., AMP, ADP, ATP, adenine-containing co-enzymes, i.e. NAD, NADP, FAD, and nucleic acids, i.e., RNA, DNA can be made for some understanding. It is estimated that the number of free adenine nucleotides and co-enzymes is in the ~ 10^5 and ~10^4 molecules per cell [34], respectively. The contribution of DNA and RNA in relative comparison is ~ 10^8-10^9 range. This would suggest adenine to mostly arise from DNA/RNA. However, the relative ionization and subsequent fragmentation to form adenine would be strongly dependent on the size of the molecule. As an example, a comparison of AMP versus ATP mass spectrum done with BAMS (data not shown) at different laser fluences indicates lower ionization efficiency for ATP compared to AMP, as the size of the molecule increases by two phosphate groups. The total ion signal for m/z=-134 and its fragments ions obtained for ATP in our studies (data not shown) is ~5x lower in comparison to AMP at 0.2 mJ laser pulse energy for a ~400μm FWHM beam. Fragmentation of DNA/RNA would similarly be expected to be less efficient in producing adenine and its fragment peaks based on its larger size. This makes us believe that AMP being the smallest adenine-containing substructure in a spore would have the highest contribution to the observed adenine related peaks. It is interesting to point out that the relative contribution of AMP to ATP in an endospore is substantially higher than a vegetative cell. For example, in *Bacillus megaterium* the ATP: (ADP+AMP) ratio is 0.22:1 and in cells 3.7:1 [34] with similar findings for *Bacillus subtilis* and *Bacillus cereus* spores. This would suggest a higher adenine related

signal in spores relative to vegetative cell and might explain the near absence of m/z=-134, -90 in vegetative cell spectrum[22].

Also confirmed is the assignment of m/z= -17, -26 and -42 as OH^-, CN^- and CNO^- based on observed mass shifts of 0,1,1 in the ^{13}C and ^{15}N media. Both CN^- and CNO^- along with PO_3^- contribute the largest ion signal in the negative spectrum. As can be seen from spectra shown in Fig. 3 nucleobases can contribute to CN^- and CNO^- ions seen in the spore spectra.

Betaine

For m/=74 our isotope results indicate a shift to m/z=75 in ^{15}N media and m/z=78 in ^{13}C media, see Fig. 2. This result is consistent with $^+N(CH_3)_4$ as well as protonated amines. However, there is no evidence in the literature of free amines being present in the spore. Quartenary ammonium salts, of the kind $^+N(CH_3)_4\,X^-$, where X^- = is a negative ion like halide, sulfate or phosphate, are also absent from the growth medium. As there is no evidence for $^+N(CH_3)_4$ to be present in the media or within the spore it is probable to be a fragment of a $^+N(CH_3)_3CH_2$- containing moiety like choline ($^+N(CH_3)_3CH_2CH_2OH$), phosphocholine (PC) headgroup of a phospholipid ($^+N(CH_3)_3CH_2CH_2OP(O)_2OCH_2$-) or trimethyl glycine ($^+N(CH_3)_3CH_2COOH$). It is unlikely for PC containing phospholipid to be the source of the observed m/z=74 fragment peak based on known lipid concentrations in *Bacillus subtilis.* *Bacillus subtilis* membranes contains [35] 70% of its lipid in the neutral lipid fraction of which phosphatidylglycerol ($CH_2(OH)CH_2(OH)CH_2OP(O^-)(O)OCH_2CH$-R(R')) is the major (70%) phospholipid and appreciable (12%) amounts of phosphatidylethanolamine(($^+N(H)_3CH_2CH_2OP(O^-)(O)OCH_2CH$-R(R'))) and no detectable amounts of phosphatidylcholine (($^+N(CH)_3CH_2CH_2OP(O^-)(O)OCH_2CH$-R(R'))). Here R(R') denotes the fatty acid chain of the phospholipid. In addition, we also ran pure standard of dioeleoyl phosphatidylcholine and did not observe any m/z=74 fragmentation peak. However, the possibility of trimethyl glycine, $^+N(CH_3)_2CH_2COO^-$, known to be present in complex growth medium as a source of m/z=74 cannot be ruled out and was further investigated. Specifically,

Dulaney et al. [36] isolated and purified betaine from yeast extract and showed that it contained about 110 μg of betaine per g of yeast extract. The mass spectrum of trimethyl glycine (common name, betaine) was collected and is shown in Fig. 4. A large fragmentation peak at m/z=-59 corresponding to $^{+}N(CH_3)_3$ is observed. There is in addition a small peak at m/z=73 and 118 corresponding to $^{+}N(CH_3)_2CH_2^{\cdot}$ and $^{+}N(CH_3)_2CH_2COOH$. This result is further examined in the presence of dipicolinic acid, which has a high uv absorption cross-section at 266nm ($\varepsilon\sim 4000$ l mol^{-1} cm^{-1}) and constitutes upto 15% of the dry weight of *Bacillus* spore. A 1:1 ratio of DPA and betaine was aerosolized and ionized. The resulting spectrum is shown in Fig. 4 along with the spectrum of pure betaine standard. The positive spectrum in addition to m/z=59, noticeably shows a large peak at m/z=74. Additional peaks corresponding to $^{+}N(CH_3)_2CH_2COOH$ are observed at 118. This result shows that DPA-betaine interaction produces a peak at m/z=74. This is most likely formed by protonation of the anionic carbon site, $^{+}N(CH_3)_2CH_2^{-}$, resulting from decarboxylation of betaine. DPA related peaks are observed in the negative spectra at m/z=-166, -122, -78 and correspond to the parent deprotonated ion and subsequent losses of 1 and 2 CO_2 groups. Control experiments with pure DPA aerosol standards did not produce any m/z=74 peak as depicted in Fig. 4. Based on evidence of betaine in complex media and its fragmentation pattern in presence of DPA we believe $[N_1C_4H_{12}]^{+}$ assignment of the m/z=74 peak in the spore mass spectrum to be $^{+}N(CH_3)_4$ from betaine. For the peak observed at m/z=59 in the spore spectrum in unlabeled media, as shown in Figs. 1 and 2, the m/z shift is by 3 carbons to m/z=62 in the ^{13}C-labeled media based on average spectra and consistent with $^{+}N(CH_3)_3$ ion. A corresponding shift by one mass unit to m/z=60 in the ^{15}N-labeled media is seen on a single spectra level but attenuated in the average spectra shown in Fig. 3. The presence of m/z=59 in the spore spectra is most likely $^{+}N(CH_3)_3$ and also consistent with the fragmentation of betaine in the presence of DPA. A small peak in the unlabeled media is also observed at m/z=118. However its assignment could not be made to determine if it is protonated betaine.

CONCLUSION

All the prominent peaks in the BAMS mass spectrum of *Bacillus atrophaeus* spore have been chemically assigned and reveal presence of purine nucleobase structures in addition to dipicolinic acid and free amino acids. This result is consistent with the high nucleobase concentrations found inside *Bacillus* spores.

The association of m/z=-173 with arginine for *Bacillus atrophaeus*, as observed in present studies, is significant as it is one of the two marker peaks whose presence in *Bacillus atrophaeus* spore spectrum might be useful to differentiate it from *Bacillus thuringiensis* spore spectrum[16]. As indicated earlier arginine is one of the predominant free amino acids in *Bacillus subtilis* spore and is present in nearly equimolar concentrations as glutamic acid. However, unlike glutamic acid, arginine is found in only certain *Bacillus* spore species [28]. In the case of *Bacillus cereus* spore free arginine is absent. To our knowledge similar data for *Bacillus thuringiensis* spore is not available for a one to one comparison with *Bacillus subtilis* spore. We can only therefore suggest that the absence of m/z=-173 peak in *Bacillus thuringiensis* spore spectra[16] is reflective of a lack of free arginine pool in *B. thuringiensis* spore.

The identification of m/z=+74 ion for *Bacillus atrophaeus* spore is also a differentiating marker peak from *Bacillus thuringiensis* for certain growth media [16]. The assignment obtained in our studies suggest it to be $^{+}N(CH_3)_4$ and be derived from betaine. Betaine can be transported into the cell via OpuA, OpuC and OpuD osmoprotectant uptake systems for osmoregulation [37]. Furthermore, it can be synthesized via the GbsA and GbsB enzymes from exogenously provided choline taken up via the OpuB and OpuC ABC transporters. Further studies that will probe the osmotic properties of *Bacillus atrophaeus* and *Bacillus thuringiensis* in high salt and glycine betaine containing growth medium are currently underway to better understand the factors controlling the presence of the fragmentation peak at m/z=+ 74 in *B. atrophaeus* and absence in *B. thuringiensis.*.

Our assignments of m/z=-173 and +74 therefore suggest that these peaks have to be used with caution for spore discrimination as they can be media dependent in the case of m/z=+74 and not unique to a single spore species as in the case of m/z=-173.

ACKNOWLEDGMENT

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REFERENCES

(1) Fenselau, C.; Demirev, P. A. *Mass Spectrometry Reviews* **2001**, *20*, 157-171.
(2) van Baar, B. L. M. *Fems Microbiology Reviews* **2000**, *24*, 193-219.
(3) Johnston, M. V.; Wexler, A. S. *Analytical Chemistry* **1995**, *67*, A721-726.
(4) Murphy, D. M.; Thomson, D. S. *Aerosol Science and Technology* **1995**, *22*, 237-249.
(5) Lazar, A.; Reilly, P. T. A.; Whitten, W. B.; Ramsey, J. M. *Environmental Science & Technology* **1999**, *33*, 3993-4001.
(6) Gard, E.; Mayer, J. E.; Morrical, B. D.; Dienes, T.; Fergenson, D. P.; Prather, K. A. *Analytical Chemistry* **1997**, *69*, 4083-4091.
(7) Hinz, K. P.; Kaufmann, R.; Spengler, B. *Analytical Chemistry* **1994**, *66*, 2071-2076.
(8) Hinz, K. P.; Kaufmann, R.; Spengler, B. *Aerosol Science and Technology* **1996**, *24*, 233-242.
(9) Goodacre, R.; Shann, B.; Gilbert, R. J.; Timmins, E. M.; McGovern, A. C.; Alsberg, B. K.; Kell, D. B.; Logan, N. A. *Analytical Chemistry* **2000**, *72*, 119-127.
(10) Snyder, A. P.; Thornton, S. N.; Dworzanski, J. P.; Meuzelaar, H. L. C. *Field Analytical Chemistry and Technology* **1996**, *1*, 49-59.
(11) Sinha, M. P.; Platz, R. M.; Vilker, V. L.; Friedlander, S. K. *International Journal of Mass Spectrometry and Ion Processes* **1984**, *57*, 125-133.
(12) Sinha, M. P.; Platz, R. M.; Friedlander, S. K.; Vilker, V. L. *Applied and Environmental Microbiology* **1985**, *49*, 1366-1373.
(13) Gieray, R. A.; Reilly, P. T. A.; Yang, M.; Whitten, W. B.; Ramsey, J. M. *Journal of Microbiological Methods* **1997**, *29*, 191-199.
(14) Parker, E. P.; Trahan, M. W.; Wagner, J. S.; Rosenthal, S. E.; Whitten, W. B.; Gieray, R. A.; Reilly, P. T. A.; Lazar, A. C.; Ramsey, J. M. *Field Analytical Chemistry and Technology* **2000**, *4*, 31-42.
(15) Stowers, M. A.; van Wuijckhuijse, A. L.; Marijnissen, J. C. M.; Scarlett, B.; van Baar, B. L. M.; Kientz, C. E. *Rapid Communications in Mass Spectrometry* **2000**, *14*, 829-833.
(16) Fergenson, D. P.; Pitesky, M. E.; Tobias, H. J.; Steele, P. T.; Czerwieniec, G. A.; Russell, S. C.; Lebrilla, C. B.; Horn, J. M.; Coffee, K. R.; Srivastava, A.; Pillai, S. P.; Shih, M. T. P.; Hall, H. L.; Ramponi, A. J.; Chang, J. T.; Langlois, R. G.; Estacio, P. L.; Hadley, R. T.; Frank, M.; Gard, E. E. *Analytical Chemistry* **2004**, *76*, 373-378.
(17) Lay, J. O. *Mass Spectrometry Reviews* **2001**, *20*, 172-194.
(18) He, L.; Murray, K. K. *Journal of Mass Spectrometry* **1999**, *34*, 909-914.
(19) Jackson, S. N.; Murray, K. K. *Analytical Chemistry* **2002**, *74*, 4841-4844.
(20) Ullom, J. N.; Frank, M.; Gard, E. E.; Horn, J. M.; Labov, S. E.; Langry, K.; Magnotta, F.; Stanion, K. A.; Hack, C. A.; Benner, W. H. *Analytical Chemistry* **2001**, *73*, 2331-2337.
(21) Steele, P. T.; Tobias, H. J.; Fergenson, D. P.; Pitesky, M. E.; Horn, J. M.; Czerwieniec, G. A.; Russell, S. C.; Lebrilla, C. B.; Gard, E. E.; Frank, M. *Analytical Chemistry* **2003**, *75*, 5480-5487.
(22) Czerwieniec, G.; Russell, S. C.; Lebrilla, C.; Gard, E.; Frank, M. *Analytical Chemistry*, Submitted.
(23) Hinds, W. C. *Aerosol Technology*, 1 ed ed.; John Wiley & Sons: New York, 1982.
(24) Perry, J. J.; Foster, J. W. *Journal of Bacteriology* **1956**, *72*, 295-300.
(25) Murrell, W. G.; Warth, A. D. In *Spores III*; American Society for Microbiology, 1965, pp 1-24.
(26) Young, E.; Fitz-James, P. C. *Journal of Cell Biology* **1962**, *12*, 115-133.
(27) Beverly, M. B.; Voorhees, K. J.; Hadfield, T. L.; Cody, R. B. *Analytical Chemistry* **2000**, *72*, 2428-2432.
(28) Nelson D. L. ; A., K. *J Biol Chem.* **1970**, *245*, 1128-1136.

(29) Russell, S. C.; Czerwieniec, G.; Lebrilla, C.; Tobias, H.; Fergenson, D. P.; Steele, P.; Pitesky, M.; Horn, J.; Srivastava, A.; Frank, M.; Gard, E. E. *Journal of the American Society for Mass Spectrometry* **2004**, *15*, 900-909.
(30) Carr, S. R.; Cassady, C. J. *Journal of Mass Spectrometry* **1997**, *32*, 959-967.
(31) Young, I. E. *Can J Microbiol.* **1959**, *5*, 197-202.
(32) Tang, T.; Rajan, K. S.; Grecz, N. *Biophys J.* **1968**, *8*, 1458-1474.
(33) Rodgers, M. T.; Campbell, S.; Marzluff, E. M.; Beauchamp, J. L. *International Journal of Mass Spectrometry and Ion Processes* **1994**, *137*, 121-149.
(34) Setlow, P.; Kornberg, A. *J Biol Chem* **1970**, *245*, 3637-3644.
(35) Clejan, S.; Krulwich, T. A.; Mondrus, K. R.; Seto-Young, D. *Journal of Bacteriology* **1986**, *168*, 334-340.
(36) Dulaney, E. L.; Dulaney, D. D.; Rickes, E. L. *Dev. Ind. Microbiol.* **1968**, *9*, 260-269.
(37) Steil, L.; Hoffmann, T.; Budde, I.; Volker, U.; Bremer, E. *Journal of Bacteriology* **2003**, *185*, 6358-6370.

FIGURE CAPTIONS

Fig. 1. Dual polarity average mass spectra of *Bacillus atrophaeus* (A, B, C 324, 420, 125 spectra, respectively) grown in Bioexpress A) unlabeled, B) ^{15}N enriched, C) ^{13}C enriched media. The inset in each panel shows the protonated arginine peak.

Fig. 2. Dual polarity average mass spectra of *Bacillus atrophaeus* grown in Bioexpress A) unlabeled, B) ^{15}N enriched, C) ^{13}C enriched media. Two mass ranges are shown, -185 to –85 on left and 55 to 95 on right. All other details are same as Fig. 1. Please see text for discussion.

Fig. 3. Dual polarity average mass spectra (200 spectra each) of nucleobase containing standards, namely A) adenine, B) guanosine 5'-diphosphate sodium salt (GDP) and C) cytidine. Spectra were obtained at 100 μJ average laser pulse energy. The encircled chemical structures in each figure represent the nucleobase sub-structures adenine (A), guanine (G) and cytosine (C), respectively. Note presence of m/z =-134, -117, -107, -90 fragment ions in adenine mass spectra, similar to that found in spore spectra.

Fig. 4. Dual polarity average mass spectra (200 spectra each) of betaine, betaine:dipicolinic acid (1:1 molar ratio), dipicolinic acid. All spectra were collected at 0.21 mJ average laser pulse energy. Note that in presence of DPA a strong m/z=74 peak is observed for betaine: DPA mixture

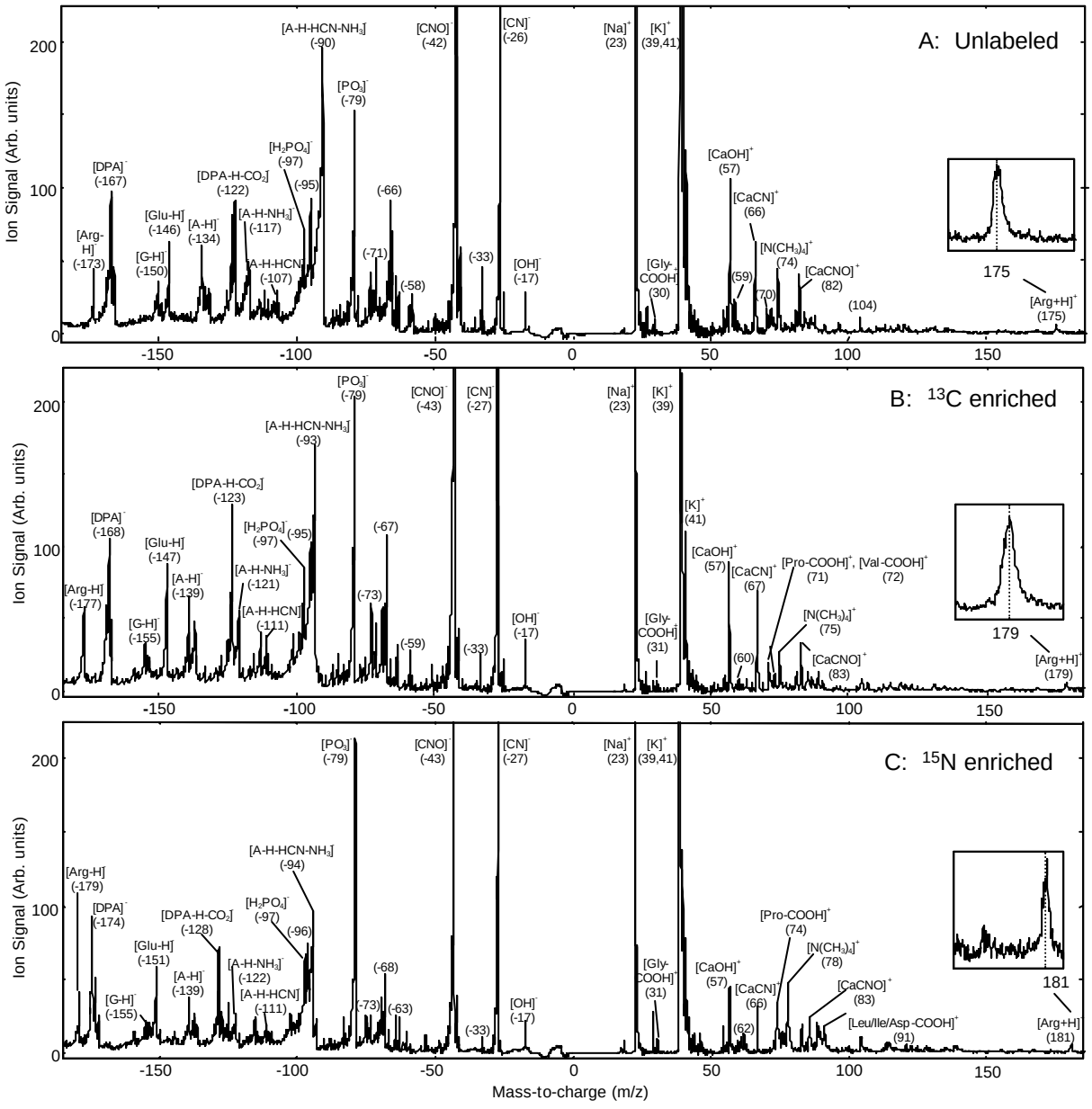
A: Unlabeled
B: ^{13}C enriched
C: ^{15}N enriched
Ion Signal (Arb. units)
Mass-to-charge (m/z)
[A-H-HCN-NH$_3$]$^-$ (-90)
[CNO]$^-$ (-42)
[CN]$^-$ (-26)
[Na]$^+$ (23)
[K]$^+$ (39,41)
[PO$_3$]$^-$ (-79)
[H$_2$PO$_4$]$^-$ (-97)
[DPA]$^-$ (-167)
[DPA-H-CO$_2$]$^-$ (-122)
[Glu-H]$^-$ (-146)
[A-H]$^-$ (-134)
[A-H-NH$_3$]$^-$ (-117)
[Arg-H]$^-$ (-173)
[G-H]$^-$ (-150)
[A-H-HCN]$^-$ (-107)
[CaOH]$^+$ (57)
[CaCN]$^+$ (66)
[N(CH$_3$)$_4$]$^+$ (74)
[CaCNO]$^+$ (82)
[Gly-COOH]$^-$ (30)
[OH]$^-$ (-17)
[Arg+H]$^+$ (175)
[DPA]$^-$ (-168)
[Glu-H]$^-$ (-147)
[A-H]$^-$ (-139)
[Arg-H]$^-$ (-177)
[G-H]$^-$ (-155)
[A-H-NH$_3$]$^-$ (-121)
[A-H-HCN]$^-$ (-111)
[DPA-H-CO$_2$]$^-$ (-123)
[K]$^+$ (41)
[CaOH]$^+$ (57)
[CaCN]$^+$ (67)
[Pro-COOH]$^+$, [Val-COOH]$^+$ (71) (72)
[N(CH$_3$)$_4$]$^+$ (75)
[CaCNO]$^+$ (83)
[Gly-COOH]$^-$ (31)
[Arg+H]$^+$ (179)
[DPA]$^-$ (-174)
[Glu-H]$^-$ (-151)
[A-H]$^-$ (-139)
[Arg-H]$^-$ (-179)
[G-H]$^-$ (-155)
[A-H-NH$_3$]$^-$ (-122)
[A-H-HCN]$^-$ (-111)
[DPA-H-CO$_2$]$^-$ (-128)
[A-H-HCN-NH$_3$]$^-$ (-94)
[Pro-COOH]$^+$ (74)
[N(CH$_3$)$_4$]$^+$ (78)
[CaOH]$^+$ (57)
[CaCN]$^+$ (66)
[CaCNO]$^+$ (83)
[Leu/Ile/Asp-COOH]$^+$ (91)
[Arg+H]$^+$ (181)

Figure 1

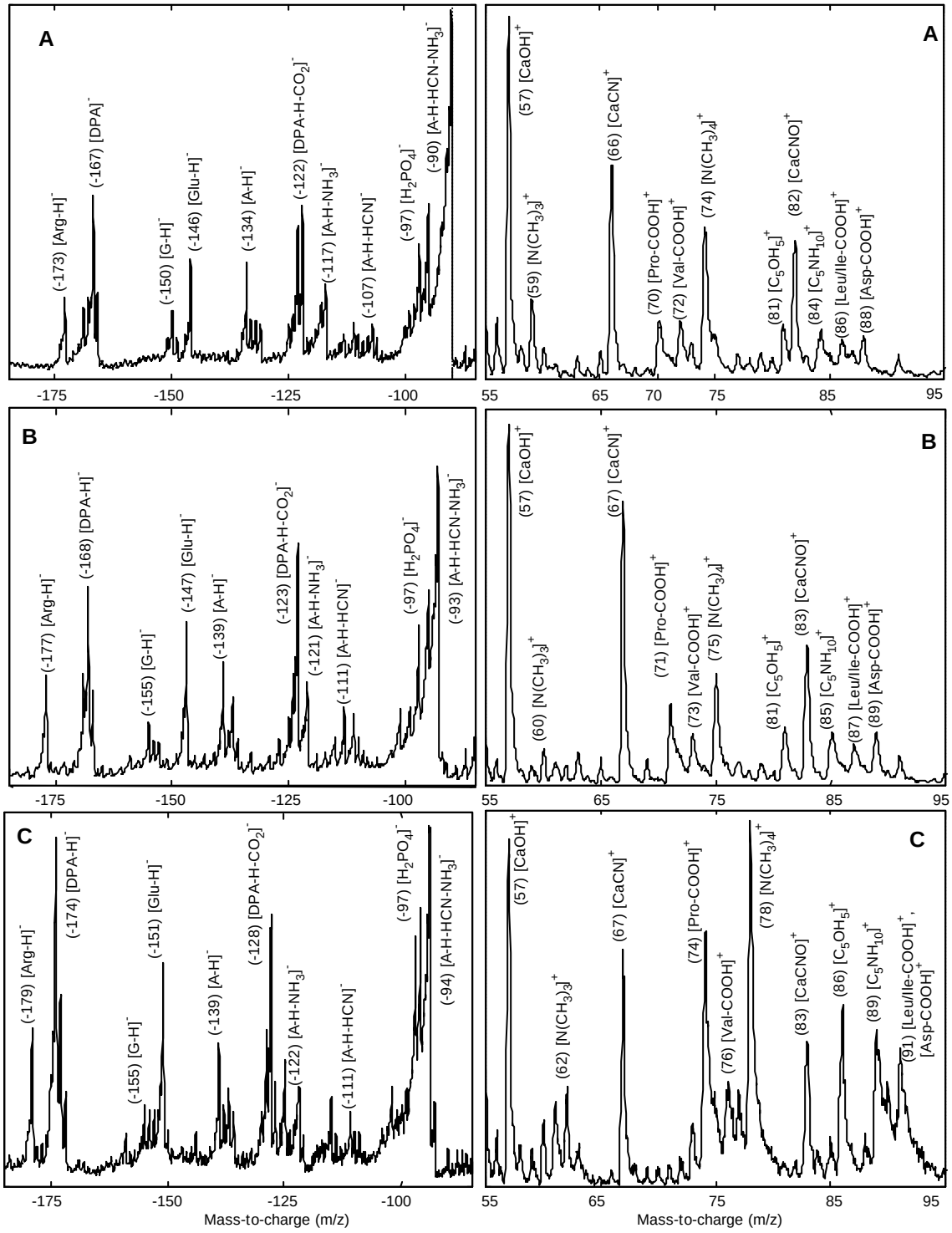
A
(-173) [Arg-H]-
(-167) [DPA]-
(-150) [G-H]-
(-146) [Glu-H]-
(-134) [A-H]-
(-122) [DPA-H-CO2]-
(-117) [A-H-NH3]-
(-107) [A-H-HCN]-
(-97) [H2PO4]-
(-90) [A-H-HCN-NH3]-
-175
-150
-125
-100
(57) [CaOH]+
(59) [N(CH3)3]+
(66) [CaCN]+
(70) [Pro-COOH]+
(72) [Val-COOH]+
(74) [N(CH3)4]+
(81) [C5OH5]+
(82) [CaCNO]+
(84) [C5NH10]+
(86) [Leu/Ile-COOH]+
(88) [Asp-COOH]+
55
65
70
75
85
95
B
(-177) [Arg-H]-
(-168) [DPA-H]-
(-155) [G-H]-
(-147) [Glu-H]-
(-139) [A-H]-
(-123) [DPA-H-CO2]-
(-121) [A-H-NH3]-
(-111) [A-H-HCN]-
(-97) [H2PO4]-
(-93) [A-H-HCN-NH3]-
(57) [CaOH]+
(60) [N(CH3)3]+
(67) [CaCN]+
(71) [Pro-COOH]+
(73) [Val-COOH]+
(75) [N(CH3)4]+
(81) [C5OH5]+
(83) [CaCNO]+
(85) [C5NH10]+
(87) [Leu/Ile-COOH]+
(89) [Asp-COOH]+
C
(-179) [Arg-H]-
(-174) [DPA-H]-
(-155) [G-H]-
(-151) [Glu-H]-
(-139) [A-H]-
(-128) [DPA-H-CO2]-
(-122) [A-H-NH3]-
(-111) [A-H-HCN]-
(-97) [H2PO4]-
(-94) [A-H-HCN-NH3]-
(57) [CaOH]+
(62) [N(CH3)3]+
(67) [CaCN]+
(74) [Pro-COOH]+
(76) [Val-COOH]+
(78) [N(CH3)4]+
(83) [CaCNO]+
(86) [C5OH5]+
(89) [C5NH10]+
(91) [Leu/Ile-COOH]+, [Asp-COOH]+
Mass-to-charge (m/z)
Mass-to-charge (m/z)

Figure. 2

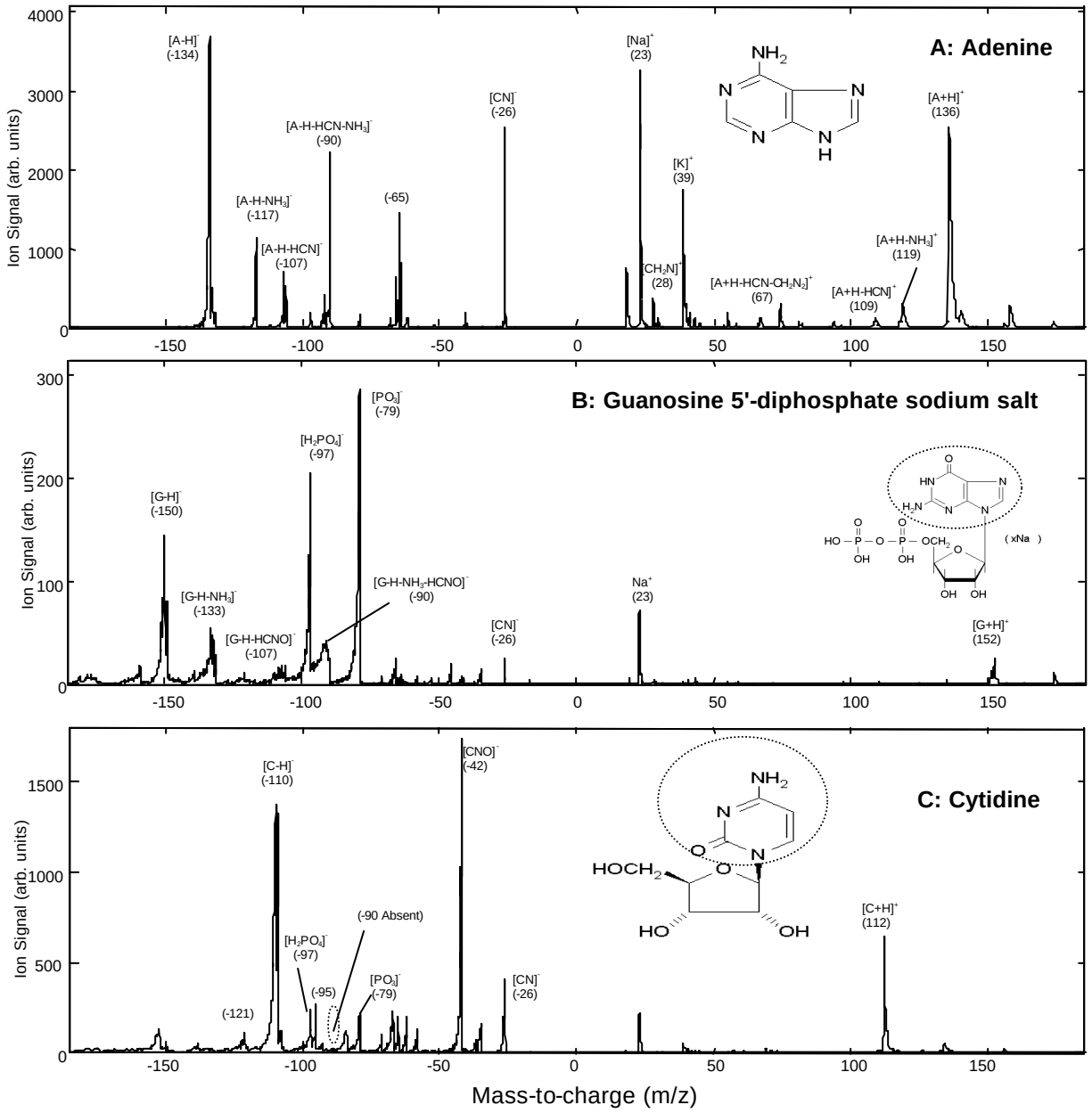
A: Adenine
[A-H]⁻ (-134)
[A-H-NH3]⁻ (-117)
[A-H-HCN]⁻ (-107)
[A-H-HCN-NH3]⁻ (-90)
(-65)
[CN]⁻ (-26)
[Na]⁺ (23)
[CH2N]⁺ (28)
[K]⁺ (39)
[A+H-HCN-CH2N2]⁺ (67)
[A+H-HCN]⁺ (109)
[A+H-NH3]⁺ (119)
[A+H]⁺ (136)
B: Guanosine 5'-diphosphate sodium salt
[G-H]⁻ (-150)
[G-H-NH3]⁻ (-133)
[G-H-HCNO]⁻ (-107)
[H2PO4]⁻ (-97)
[PO3]⁻ (-79)
[G-H-NH3-HCNO]⁻ (-90)
[CN]⁻ (-26)
Na⁺ (23)
[G+H]⁺ (152)
(xNa)
C: Cytidine
[C-H]⁻ (-110)
(-121)
[H2PO4]⁻ (-97)
(-95)
(-90 Absent)
[PO3]⁻ (-79)
[CNO]⁻ (-42)
[CN]⁻ (-26)
[C+H]⁺ (112)
Ion Signal (arb. units)
Mass-to-charge (m/z)

Figure. 3

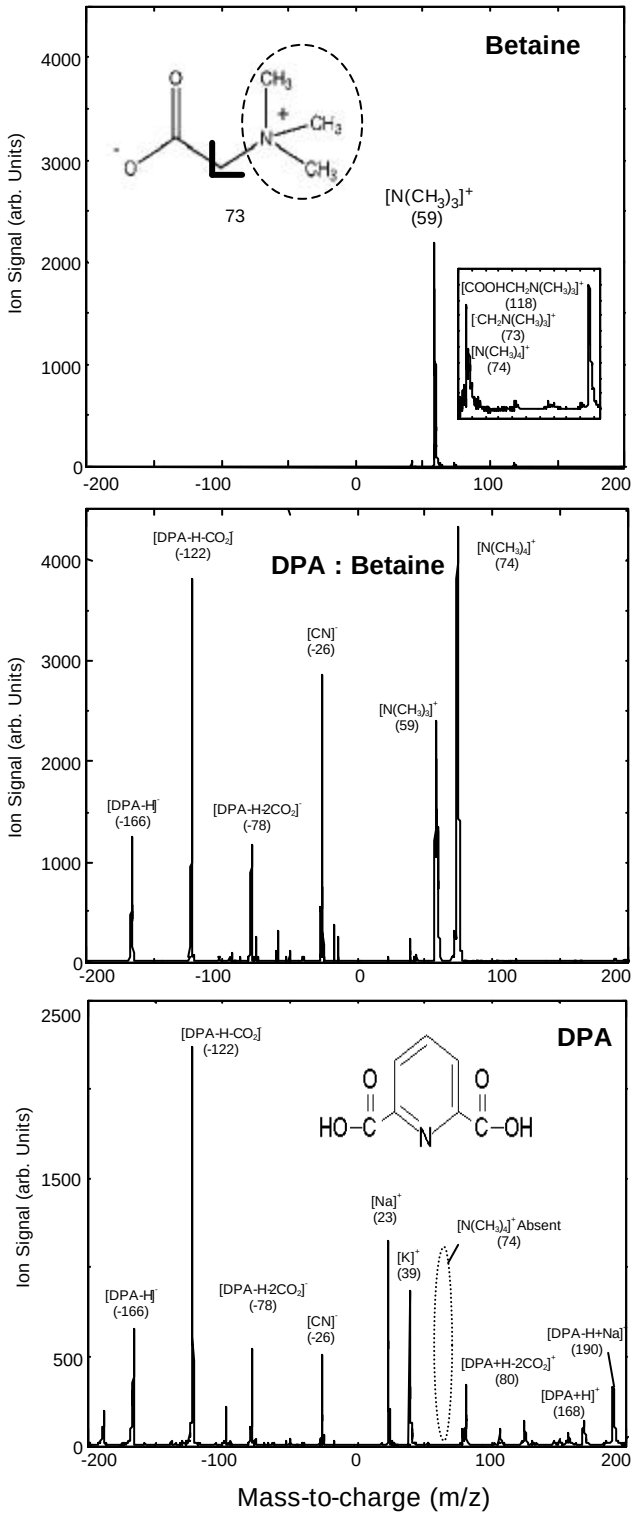
Betaine
73
[N(CH3)3]+
(59)
[COOHCH2N(CH3)3]+
(118)
[CH2N(CH3)3]+
(73)
[N(CH3)4]+
(74)
DPA : Betaine
[DPA-H-CO2]-
(-122)
[N(CH3)4]+
(74)
[CN]-
(-26)
[N(CH3)3]+
(59)
[DPA-H]-
(-166)
[DPA-H-2CO2]-
(-78)
DPA
[Na]+
(23)
[N(CH3)4]+ Absent
(74)
[K]+
(39)
[DPA+H-2CO2]+
(80)
[DPA-H+Na]-
(190)
[DPA+H]+
(168)
Ion Signal (arb. Units)
Mass-to-charge (m/z)

Figure. 4